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The purpose of this project is to generate monoclonal antibodies to receptor molecules for the angiogenic growth factor VEGF. These antibodies will be tested for their potential as inhibitors of VEGF-stimulated angiogenesis, which is thought to play an important role in the development of breast cancer. A number of hybridoma fusions have been done, but screening has been delayed. Research defining the VEGF binding site of the Flt-1 receptor is in press; this work will aid in the selection of receptor fragments for immunization. Research elucidating two signaling pathways required for VEGF-stimulated endothelial cell proliferation has been published; a conclusion of that study is that therapeutic intervention of VEGF-induced angiogenesis should focus on early signaling events.

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FOREWORD

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J. Barry [illegible] 8/29/99

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Introduction

The survival and growth of solid tumors is dependent on the neovascularization of the growing tumor mass. Prior to the vascularization of a tumor, a subpopulation of tumor cells acquire an angiogenic phenotype characterized by the production of polypeptide growth factors that stimulate endothelial cell proliferation, migration and differentiation. These tumor-derived angiogenic factors induce the growth of blood vessels into the tumor from pre-existing blood vessels. Thus, the process of tumor angiogenesis represents an attractive target for the development of new cancer therapeutic agents. We are generating neutralizing monoclonal antibodies to receptor molecules for vascular endothelial growth factor, an angiogenic factor that is widely expressed by breast tumors. We will test the antibodies for the ability to block VEGF-induced endothelial cell proliferation and capillary growth in vitro and tumor formation by breast carcinoma cells in vivo.

Body of Report

Specific Aim 1: Tasks 1-3

Vascular endothelial cell growth factor (VEGF) stimulates human endothelial cell proliferation by binding to two cell surface receptor molecules: Flt-1 (fms-like tyrosine kinase) and KDR (kinase insert domain-containing receptor) (1,2). Davis-Smyth, et al. (3) have used domain swapping between Flt-1 and a related receptor molecule, Flt-4, that does not bind VEGF to localize the major VEGF binding region of Flt-1 to the second extracellular immunoglobulin-like loop. As part of this project, we have examined cell-bound receptor chimeras consisting of fragments of the extracellular region of Flt-1 and a non-related cell surface molecule embigin to further characterize the VEGF-binding region of Flt-1 (4, Appendix 1). Our results indicate that either immunoglobulin-like loop 1 or 3 of Flt-1 is required with loop 2 for high affinity VEGF binding. These findings in conjunction with the work of others (5,6) localize the VEGF binding region of Flt-1 to extracellular immunoglobulin-like loops 1 to 3. We will incorporate these results into our receptor immunization protocol.

We have done four hybridoma fusions with spleen cells from mice immunized with insect cells expressing recombinant human Flt-1. The fusion products were allowed to recover in culture for several days, and then they were cryopreserved. These fusion products will be screened for neutralizing antibody activity by competitive binding with radioactive VEGF once an institutional license for the use of radioactive materials is granted by the Nuclear Regulatory Commission. In anticipation of screening, we have constructed and expressed mutant forms of VEGF that bind selectively to Flt-1 or KDR based on an analysis of receptor binding regions of VEGF by Keyt, et al. (7). These mutant VEGFs will allow us to screen for antibodies to a single receptor type using human endothelial cells that express both receptor types.

Specific Aim 2: Task 7

We have done a pilot experiment aimed at developing an in vitro microvessel growth assay. A piece of human umbilical cord was cut into small fragments which were plated on a collagen-coated substratum in several different media. Initially DME/F12 nutrient medium was supplemented with either 10% fetal bovine serum, 10 ng/ml fibroblast growth factor-2 (FGF-2), 10 ng/ml VEGF or FGF-2 and VEGF. After a growth phase in which cells migrated out of the tissue fragments and proliferated on the substratum, the nutrient medium supplemented with the purified growth factors was changed to MCDB 153, which possesses a low calcium concentration. A large percentage of cells died in the new medium, which was consistent with the inability of fibroblast cells to grow under conditions of low calcium (8). The remaining cells had the morphological characteristics of

endothelial cells, but they have not yet been tested for biochemical markers of endothelial cells. These cells were not embedded in a collagen gel and therefore did not form tube structures.

The strategy of inhibiting the angiogenic activity of VEGF at the level of ligand-receptor interactions at the cell surface rather than at the level of intracellular signaling pathways is supported by our study of intracellular pathways that are activated in endothelial cells during a mitogenic response to VEGF (9, Appendix 2). We found that the mitogen-activated protein (MAP) kinases ERKs 1 and 2, and p38 MAP kinase but not Jun N-terminal kinase (JNK) were activated in response to VEGF. In addition phosphatidylinositol 3-kinase (PI 3-kinase) signaling through p70 S6 kinase was activated. By using specific inhibitors of these kinases, we showed that activation of ERKs 1 and 2 and the PI 3-kinase/p70 S6 kinase pathway were both required for endothelial cells to proliferate in response to VEGF. However, these pathways were not uniquely activated by VEGF but were also activated by receptors for epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2). This observation of convergent growth factor signaling in endothelial cells indicates that therapeutic strategies aimed at inhibiting intracellular signaling components will not be specific for responses to VEGF. This conclusion is a compelling argument for focusing on proximal steps in VEGF signaling to inhibit angiogenesis.

Progress in the first year of this grant has been impacted by the relocation of the Principal Investigator from the Adirondack Biomedical Research Institute to the American Type Culture Collection, a not for profit bioscience institution in Manassas, VA., where the P.I. occupies a laboratory on the Prince William Campus of George Mason University. The University is in the process of applying for an amendment to its radioactive materials use license that will cover the new labs on the Prince William Campus. The P.I. has not requested the transfer of this grant to the ATCC while he is unable to use radioisotopic methods to screen hybridomas, and no charges have been made to the grant for work done at the ATCC.

Key Research Accomplishments

- Characterization of the minimal high affinity VEGF binding site on the Flt-1 receptor

- Identification of required signaling pathways in VEGF-induced cell proliferation

Reportable Outcomes

Publications

1. Herley, M.T., Yu, Y., Whitney, R.G., and Sato, J.D. (1999) Characterization of the VEGF binding site on the Flt-1 receptor. Biochem. Biophys. Res. Commun. (In press).

2. Yu, Y., and Sato, J.D. (1999) MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. J. Cell. Physiol. 178: 235-246.

Conclusions

The major conclusion of the research done thus far is that because signaling pathways initiated by different growth factors converge or overlap, therapeutic interventions of angiogenesis or other growth factor-induced processes should target early events in signaling cascades in order to achieve the greatest degree of specificity.

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9. Yu, Y., and Sato, J.D. (1999) MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. J. Cell. Physiol. 178: 235-246.

Appendices

Appendix 1

Characterization of the VEGF binding site on the Flt-1 receptor. Biochem. Biophys. Res. Commun. (In press) (1999).

Appendix 2

MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. J. Cell. Physiol. 178: 235-246 (1999).

Characterization of the VEGF binding site on the Flt-1 receptor

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ABSTRACT

The angiogenic growth factor VEGF binds to the receptor tyrosine kinases Flt-1 and KDR/Flk-1. Immunoglobulin (Ig)-like loop-2 of Flt-1 is involved in binding VEGF, but the contribution of other Flt-1 Ig-loops to VEGF binding remains unclear. We tested the ability of membrane-bound chimeras between the extracellular domain of Flt-1 and the cell adhesion molecule embigin to bind VEGF. VEGF bound as well to receptors containing Flt-1 loops 1-2 or 2-3 as it did to the entire Flt-1 extracellular domain. Chimeras containing only loop-2 of Flt-1 bound VEGF with 22-fold lower affinity. We conclude that high affinity VEGF binding requires Ig-like loop-2 plus either loop-1 or loop-3. In addition, Flt-1 amino acid residues Arg-224 and Asp-231 were not essential for high affinity binding of VEGF to membrane-bound Flt-1.

JOURNAL OF CELLULAR PHYSIOLOGY 178:235–246 (1999)

MAP Kinases, Phosphatidylinositol 3-Kinase, and p70 S6 Kinase Mediate the Mitogenic Response of Human Endothelial Cells to Vascular Endothelial Growth Factor

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Although the significance of vascular endothelial growth factor (VEGF) and its receptors in angiogenesis is well established, the signal transduction cascades activated by VEGF and their involvement in mediating the mitogenic response of endothelial cells to VEGF are incompletely characterized. Here we demonstrate that VEGF activates mitogen-activated protein (MAP) kinases, including the extracellular signal-regulated protein kinase (ERK) and p38 MAP kinase, phosphatidylinositol 3-kinase (PI 3-kinase), and p70 S6 kinase in human umbilical vein endothelial cells (HUVEC). The activation of these enzymes was assayed by kinase phosphorylation and by kinase activity towards substrates. Studies with PI 3-kinase inhibitors revealed that activation of p70 S6 kinase was mediated by PI 3-kinase. Selective inhibition of ERK, PI 3-kinase, and p70 S6 kinase with the inhibitors PD098059, LY294002, and rapamycin, respectively, inhibited VEGF-stimulated HUVEC proliferation. In marked contrast, the p38 MAP kinase inhibitor SB203580 not only failed to inhibit but actually enhanced HUVEC proliferation; this effect was associated with the phosphorylation of Rb protein. Rb phosphorylation resulted from a decrease in the level of the cdk inhibitor $p27^{Kip1}$. These results indicate that the activities of ERK, PI 3-kinase, and p70 S6 kinase are essential for VEGF-induced HUVEC proliferation. p38 MAP kinase suppresses endothelial cell proliferation by regulating cell-cycle progression. J Cell Physiol 178:235–246, 1999. © 1999 Wiley-Liss, Inc.

Vascular endothelial growth factor (VEGF) is a key regulator in both physiological and pathological angiogenesis (Connolly et al., 1989; Kim et al., 1993; Carmeliet et al., 1996). VEGF is distinct from other angiogenic factors in that it is an endothelial cell–specific mitogen (Leung et al., 1989; Keck et al., 1989; Myoken et al., 1991). VEGF plays a critical role in the regulation of endothelial cell proliferation, which is a major step in angiogenesis (Risau, 1997). The biological effects of VEGF are mediated by the specific receptor tyrosine kinases KDR/Flk1 and Flt1, which are composed of seven extracellular Ig-like domains, a single transmembrane sequence, and an intracellular region containing split tyrosine kinases (Mathews et al., 1991; de Vries et al., 1992; Terman et al., 1992; Quinn et al., 1993). Although it is known that VEGF receptors undergo autophosphorylation in response to VEGF (Myoken et al., 1991; Quinn et al., 1993; Seetharam et al., 1995), the subsequent biochemical events mediating the mitogenic response of endothelial cells to VEGF have not been completely characterized. Previous studies have demonstrated the VEGF-dependent phosphorylation of several cytoplasmic signaling proteins, including phosphatidylinositol 3-kinase (PI 3-kinase), PLCγ, Ras GTPase-activating protein, the adapter proteins NcK (Guo et al., 1995) and Shc (Kroll and Waltenberger, 1997), and focal adhesion kinase (Abedi and Zachary, 1997). These proteins can potentially participate in receptor signaling pathways.

In mammalian cells, ligand binding to receptor tyrosine kinases trigger the activation of downstream signaling enzymes, including MAP kinase, PI 3-kinase, p70 S6 kinase, and PLCγ (Marshall, 1995). Activation of these signaling intermediates transduces extracellular signals to the nucleus and ultimately regulates gene expression and cellular responses such as cell proliferation, migration, differentiation, and apoptosis. This model has not been completely tested with respect to VEGF-receptor signaling. The goal of the present study was to characterize the signaling enzymes that mediated the mitogenic response of endothelial cells to

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